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A Secretory Leukocyte Protease Inhibitor Variant with Improved Activity against Lung Infection

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Keywords: protease inhibitor, lung, inflammation

Abstract

Secretory leukocyte protease inhibitor (SLPI) is an important respiratory tract host defence protein which is proteolytically inactivated by excessive neutrophil elastase (NE) during chronic *Pseudomonas* infection in the cystic fibrosis (CF) lung. We generated two putative NE-resistant variants of SLPI by site-directed mutagenesis, SLPI-A16G and SLPI-S15G-A16G, with a view to improving SLPI's proteolytic stability. Both variants showed enhanced resistance to degradation in the presence of excess NE as well as CF patient sputum compared to SLPI-wild type (SLPI-WT). The ability of both variants to bind bacterial LPS and interact with NF- κ B DNA binding sites was also preserved. Finally, we demonstrate increased anti-inflammatory activity of the SLPI-A16G protein compared to SLPI-WT in a murine model of pulmonary *Pseudomonas* infection. This study demonstrates the increased stability of these SLPI variants compared to SLPI-WT and their therapeutic potential as a putative anti-inflammatory treatment for CF lung disease.

Introduction

Chronic lung infection, inflammation, and gradual destruction of the lung are characteristic clinical features of cystic fibrosis (CF). This gradual loss of lung function is fatal to most CF patients as over 95% die of respiratory failure. Neutrophils play a leading role in the destruction of the lung tissue in CF¹⁻³. Infection by particularly resistant pathogens such as *Pseudomonas aeruginosa*⁴ causes impairment of host immune defense mechanisms triggering a sustained pro-inflammatory environment and the recruitment of neutrophils in high numbers to the CF airways⁵. As a consequence of this accumulation of neutrophils, elevated levels of proteases, such as neutrophil elastase (NE), are found in the CF airways^{6,7}. NE is regarded as a key driver of lung destruction^{8,9} and inflammation especially during chronic *P. aeruginosa* infection in the CF lung^{10,11}.

Consequently, the development of NE inhibitors has been a major research focus. Small molecule inhibitors such as L-658,758¹² as well as protein-based inhibitors of NE such as EPI-hNE4/DX-890^{13,14} have been developed for this purpose. Naturally occurring inhibitors such as α -1 antitrypsin, SLPI, elafin and monocyte/neutrophil elastase inhibitor (MNEI) have also attracted a lot of interest. They have a broad protease inhibition spectrum, which is clearly beneficial in the context of CF lung disease where many different proteases are involved in the degradation of the lung tissue. In addition, some of these natural antiproteases have other properties which could contribute to the reduction of lung tissue degradation. SLPI and elafin for instance have been demonstrated to possess antibacterial as well as anti-inflammatory properties¹⁵⁻¹⁸.

One major limitation to the use of natural antiproteases as therapeutic drugs for the treatment of CF lung disease is their susceptibility to cleavage by proteases^{19,20}. In the CF lung where protease levels are very high, many endogenous protease inhibitors are found in

a cleaved, inactive form ^{10,11}. Engineering antiproteases to make them resistant to proteolytic degradation could be a way of enhancing their therapeutic profile to a sufficient level to turn them into marketed drugs.

SLPI, a member of the lung antiprotease screen particularly abundant in the upper airways and a potent inhibitor of NE ²¹, was demonstrated to be cleaved by NE in its N-terminal domain at positions Ser¹⁵-Ala¹⁶ and Ala¹⁶-Gln¹⁷, in the lungs of CF patients infected with *P. aeruginosa* ¹⁰. In this study, we have developed new ways to improve SLPI's stability which may improve its therapeutic potential for the treatment of CF lung inflammation.

Results

Design of SLPI variants resistant to cleavage by neutrophil elastase

The determination of the NE cleavage site at positions Ser¹⁵-Ala¹⁶ and Ala¹⁶-Gln¹⁷ in the amino acid sequence of human SLPI-WT by Weldon *et al.*¹⁰, paved the way for the design of SLPI variants resistant to degradation by this protease. Taking advantage of the key role played by the amino acid at the P1 position of a protease cleavage site in the determination of the susceptibility of the substrate to cleavage by the protease²², variants resistant to cleavage by a given protease can be conveniently engineered by a simple change of the amino acid at the P1 position of the cleavage site²³. The MEROPS database²⁴ provides a list of NE substrates and their amino acid composition at the cleavage site. This information was used to select the amino acids best suited to replace those at the P1 positions of the NE cleavage sites in the SLPI sequence. Two amino acids, tryptophan and histidine, were never found at the P1 position of the cleavage site in the 482 NE substrates listed in the MEROPS database. However, we selected glycine (only found to be present at the P1 of a NE substrate in two occurrences; Figure 1A) to replace serine and alanine at the P1 position of the two NE cleavage sites in the SLPI amino acid sequence, owing to a reduced likelihood of disruption of the overall protein conformation with this amino acid. Two SLPI variants were engineered by site-directed mutagenesis. As illustrated in Figure 1B, the first variant (SLPI-A16G) has a glycine residue instead of an alanine residue at the P1 position of the preferred Ala¹⁶-Gln¹⁷ NE cleavage site¹⁰. In the second variant (SLPI-S15G-A16G), the amino acids at the P1 positions of the two NE cleavage sites were replaced by glycine residues.

Expression and purification of recombinant SLPI-WT and SLPI-A16G and SLPI-S15G-A16G variants

SLPI-WT and its SLPI-A16G and SLPI-S15G-A16G variants were expressed in a bacterial system, using M15 [pREP4] *Escherichia coli* cells as a host and pQE30 as an expression vector. In this system, the recombinant proteins were expressed with an N-terminal His₆-tag for convenient purification by immobilized metal ion affinity chromatography (IMAC). After purification, all three recombinant proteins were isolated in similar yields (1.3 – 1.9 mg per liter of culture). SLPI-WT and its two variants gave bands of the expected molecular weight (ca. 13 kDa) when analysed by SDS-PAGE and could be detected by both anti-SLPI and anti-His antibodies by Western blotting, confirming the integrity of the proteins (Figure 2). The purity of all three proteins was confirmed by SDS-PAGE analysis followed by staining with Coomassie Brilliant Blue (Figure 2A). Identity was also confirmed by Western blotting with anti-SLPI and anti-His antibodies (Figure 2B-C).

Inhibition of NE by SLPI-A16G and SLPI-S15G-A16G variants

To determine if the mutations engineered in SLPI amino acid sequence had any effect on its antiprotease activity, an NE activity assay was carried out where recombinant SLPI-WT, SLPI-A16G and SLPI-S15G-A16G were incubated at 37°C with NE in the presence of a fluorogenic peptide substrate specific for NE. All proteins were able to fully inhibit NE in similar molar ratios (data not shown).

Interaction of SLPI variants with NF-κB DNA binding sites

The ability to bind and block NF-κB DNA binding sites in the promoter regions of target genes involved in the inflammatory response is an important mechanism through which SLPI exerts its anti-inflammatory properties²⁵. Previous work has demonstrated that this ability is abrogated following cleavage of SLPI by NE¹⁰. To determine if the mutations engineered in SLPI amino acid sequence had any effect on its ability to bind to NF-κB DNA binding sites,

SLPI-WT, SLPI-A16G and SLPI-S15G-A16G variants were incubated with an NF- κ B consensus oligonucleotide and the formation of SLPI:DNA complexes was monitored by EMSA. Bands corresponding to SLPI:DNA complexes were detected for all three recombinant proteins, suggesting that the variants retained the ability to bind to NF- κ B DNA binding sites and interfere with pro-inflammatory pathways (Figure 3).

Binding of SLPI variants to *P. aeruginosa* LPS

The binding of SLPI to bacterial LPS and the possible ensuing disruption of the bacterial membrane are thought to be responsible for SLPI anti-bacterial properties²⁶. This ability to bind LPS is lost upon cleavage of SLPI by NE¹⁰. To see if the mutations engineered in the SLPI amino acid sequence could have affected its ability to bind bacterial LPS, the binding of all three recombinant proteins to *P. aeruginosa* LPS was tested by indirect ELISA. SLPI-A16G and SLPI-S15G-A16G variants as well as SLPI-WT all showed a dose-dependent binding to *P. aeruginosa* LPS, suggesting that the mutations in the SLPI amino acid sequence did not alter its capacity to interact with bacterial LPS (Figure 4).

Effects of excesses of NE and CF sputum on recombinant SLPI-WT and SLPI-A16G and SLPI-S15G-A16G variants

To determine if the SLPI variants were resistant to degradation by NE, equal amounts of active recombinant SLPI-WT, SLPI-A16G and SLPI-S15G-A16G were incubated with a 10-fold molar excess of NE at 37°C and samples were taken at various timepoints and analysed by SDS-PAGE and Western blotting with both anti-SLPI and anti-His antibodies. Recombinant SLPI-WT was quickly degraded in the presence of an excess of NE (Figure 5A). Both SLPI-

A16G and SLPI-S15G-A16G mutants proved much more resistant to degradation by NE with only partial cleavage of SLPI-S15G-A16G evident (Figure 5A). To further demonstrate the clinical relevance of the two SLPI variants, equal amounts of active recombinant SLPI-WT, SLPI-A16G and SLPI-S15G-A16G were incubated with CF patient sputum. As in experiments involving incubation with purified NE, recombinant SLPI-WT was quickly degraded (Figure 5B). The two SLPI variants proved again more resistant to degradation by CF patient sputum than recombinant SLPI-WT and significant amounts of protein could be still be detected following incubation (Figure 5B). The density of each SLPI band was evaluated using densitometry and confirmed a greater decrease of the SLPI-WT protein compared to SLPI-A16G and SLPI-S15G-A16G following incubation with NE (Figure 5C) or CF sputum (Figure 5D).

Effect of SLPI-WT and SLPI-A16G in an in vivo model of pulmonary infection

We have previously demonstrated significant cleavage of SLPI by NE in the lungs of CF patients infected with *P. aeruginosa*¹⁰. Following on from our experiments in Figure 5 demonstrating increased resistance of SLPI-A16G to cleavage by NE and CF sputum compared to SLPI-WT, we tested the effect of both proteins in an *in vivo* model of pulmonary infection induced by instillation of a clinical strain of *P. aeruginosa* isolated from the lungs of a patient with CF. SLPI-A16G was chosen over SLPI-S15G-A16G as it appeared to be more stable in incubations with NE and pooled CF sputum. Although we did not demonstrate a reduction in bacterial counts in the mice treated with SLPI-WT and SLPI-A16G versus untreated mice (data not shown), we did observe a significant decrease in neutrophil recruitment to the lungs of mice treated with SLPI-A16G versus mice instilled with *P. aeruginosa* alone (Figure 6A). In contrast, mice treated with SLPI-WT did not exhibit a

decrease in lung neutrophil recruitment (Figure 6A). To explain this decrease in neutrophil recruitment, we measured serum levels of the neutrophil chemoattractant KC (Figure 6B). KC levels were significantly decreased in the serum of mice treated with SLPI-A16G but not in the serum of mice treated with SLPI-WT. Other cytokines/chemokines were measured in lung homogenate (supplementary Figure 1 - $\text{TNF}\alpha$, $\text{IL1}\beta$, IL6, KC and MIP-2) and serum (supplementary Figure 2 – $\text{IL1}\beta$, IL6 and MIP-2) but were shown not to be significantly decreased in *Pseudomonas*-infected SLPI-WT versus SLPI-AG groups.

Discussion

SLPI has previously been used in a clinical study in CF patients leading to reduced NE activity and neutrophil levels in the lungs of these patients²⁷. However, we have previously shown that during *Pseudomonas* infection in the CF lung, elevated levels of NE are responsible for the proteolytic cleavage of SLPI¹⁰. Two NE cleavage sites in SLPI were identified between residues Ser¹⁵-Ala¹⁶ and between Ala¹⁶-Gln¹⁷. Based on this information, we designed variants of SLPI that we hypothesised would be more resistant to degradation by NE, speculating that this could perhaps improve SLPI's potential as a therapeutic. We introduced substitutions into the SLPI sequence at the NE cleavage sites to reduce cleavage susceptibility. The resulting proteins (SLPI-A16G and SLPI-S15G-A16G) were successfully more resistant to cleavage by NE and *Pseudomonas*-infected CF sputum, and, in addition retained many of the features of native SLPI.

With the use of the *Pseudomonas* infection model of lung inflammation, we have demonstrated that the SLPI-A16G variant displayed enhanced anti-inflammatory properties when compared to the native SLPI-WT protein, as indicated by a significant reduction in inflammatory cells within the lungs, specifically, neutrophils. Neutrophilic influx to the respiratory tract is characteristic of the initial stages of acute pulmonary infection and is associated with disruption of the alveolar-capillary barrier and lung tissue damage^{28,29}. Bacterial infection is known to upregulate the expression of key neutrophil chemokines including KC and MIP-2. However, in this current investigation, we noted that only KC levels were significantly reduced in mice treated with the SLPI-A16G mutant which was not the case in mice treated with SLPI-WT. Other key chemokines and cytokines (TNF- α , MIP-2, IL-6, IL-1 β) were not reduced in mice treated with either SLPI-A16G or SLPI-WT (data not shown). We postulate that this reduction in KC levels in mice treated with SLPI-A16G may be related

to the enhanced cleavage resistance and therefore sustained anti-inflammatory effect of this variant compared to the native SLPI-WT protein.

We have previously demonstrated that native recombinant SLPI can inhibit LPS-induced IL-8 production by macrophages, but in our *in vivo* infection model this anti-inflammatory effect of native SLPI is not evident, most likely due to the proteolytic degradation of SLPI by increased levels of NE. We have observed similar effects when we mutated the SLPI-related WFDC protein, elafin, to enhance its stability. In that study, we found that a more NE-resistant variant of elafin (GG-elafin) could also significantly reduce neutrophil recruitment in a mouse model of LPS-induced lung inflammation compared to wild type elafin³⁰. However, in contrast to the current SLPI study, we found that GG-elafin did not reduce KC levels. This may be related to the fact that SLPI and elafin have different anti-inflammatory effects. However, it may also be due to the use of different models of inflammation in the two studies – *Pseudomonas*-induced inflammation versus LPS-induced inflammation.

One of the limitations of this study is the use of an acute lung infection model to replicate what happens in a chronic lung infection-based disease such as CF. Future studies may involve evaluation of SLPI-A16G and SLPI-WT in a relevant chronic inflammation model such as the β ENaC-transgenic model which reproduces many of the features of CF-like lung disease³¹. Another unexpected effect of SLPI-WT and SLPI-A16G in this study was the absence of any reduction in CFUs in the *in vivo* model of infection. SLPI has previously been shown to display antibacterial activity against Gram negative bacteria such as *P. aeruginosa*³². However, to our knowledge, SLPI's antibacterial activity in *in vivo* models of lung infection has not been evaluated and there may be no effect of SLPI against bacteria *in vivo*.

Overall, our findings indicate that the SLPI-A16G and SLPI-S15G-A16G variants that we have engineered are more resistant to protease degradation, whilst retaining antiprotease and anti-inflammatory properties similar to the SLPI-WT protein. These SLPI variants may hold enhanced therapeutic potential. In particular we have shown that SLPI-A16G may be of therapeutic benefit in reducing the potentially damaging inflammatory response to infection in the lung, as demonstrated by its increased effectiveness in a murine model of pulmonary infection compared to the proteolytically sensitive SLPI-WT protein.

Methods

Cloning of SLPI variants

pET32c-SLPI was a kind gift from Dr. André Cantin (University of Sherbrook, Canada). The DNA sequence encoding for human wild-type SLPI was cloned into the KpnI and HindIII restriction sites of the pQE30 expression vector (Qiagen, Manchester, UK) and this plasmid is referred to as pQE30-SLPI-WT. For each SLPI variant, the synthesis of the mutant strand was performed by PCR using 25 ng of pQE30-SLPI-WT plasmid, 125 ng of the relevant forward and reverse primers (Table 1), 2.5 U of *PfuTurbo* DNA polymerase and 1 µl of dNTP mix and 5 µl of 10 x reaction buffer as required in a final volume of 50 µl as per (Quik-site Directed Mutagenesis kit, Agilent Technologies LDA UK Limited, Stockport, UK). This mix was then subjected to PCR under the conditions of 30 s at 95°C; 16 cycles of 30 s at 95°C, 1 min at 55°C, and 4 min 30 s at 68°C. After cooling down the reaction to 37°C, 10 U of DpnI (New England Biolabs, Hitchin, UK) were added to the PCR mix and the parental strands were digested at 37°C for 1 h. The digestion products were then used to transform *E. coli* XL1-Blue supercompetent cells (Agilent Technologies LDA UK Limited, Stockport, UK). The success of the site-directed mutagenesis was verified by DNA sequencing and, for each SLPI variant, a single clone was used for all subsequent experiments. The plasmids encoding for the SLPI variants SLPI-A16G and SLPI-S15G-A16G are referred to as pQE30-SLPI-A16G and pQE30-SLPI-S15G-A16G, respectively.

Expression of SLPI-WT, SLPI-A16G and SLPI-S15G-A16G variants in M15 [pREP4] E. coli

M15 [pREP4] *E. coli* cells (Qiagen, Manchester, UK) were transformed with the pQE30-SLPI-WT, pQE30-SLPI-A16G and pQE30-SLPI-S15G-A16G plasmids and grown overnight with shaking at 37°C in Luria-Bertani (LB) medium (5 ml) supplemented with 100 µg/ml ampicillin

and 25 µg/ml kanamycin (Sigma-Aldrich, Dorset, UK). These overnight cultures (2.5 ml) were used to inoculate LB (500 ml) supplemented with 100 µg/ml ampicillin and 25 µg/ml kanamycin and pre-warmed to 37°C. The cultures were grown with shaking at 37°C until OD₆₀₀ reached 0.6. Expression of the recombinant proteins was then induced with IPTG (1 mM final concentration). The cultures were incubated at 37°C with shaking for another 4 h before the cells were harvested by centrifugation (4,000 rpm for 30 min at 4°C). Cells pellets awaiting purification were stored at - 80°C.

Purification of SLPI-WT, SLPI-A16G and SLPI-S15G-A16G

Cell pellets were thawed on ice then resuspended in lysis buffer (40 ml) containing 8 M urea, 1 mM β-mercaptoethanol, 500 mM NaCl, 5 mM imidazole and 20 mM sodium phosphate, pH 8.0. The cells were lysed for 2 h at room temperature with shaking. The cell lysates were clarified by centrifugation (4,500 x g for 1 h) followed by filtration of the supernatants through 0.2 µm filter disks. HiTrap™ Chelating HP columns (1 ml) (GE Healthcare Life Sciences, Little Chalfont, UK) were mounted on AKTAprime™ chromatography systems (GE Healthcare Life Sciences, Little Chalfont, UK), charged with Ni²⁺ ions and equilibrated with a buffer containing 8 M urea, 1 mM β-mercaptoethanol, 500 mM NaCl, 5 mM imidazole and 20 mM sodium phosphate, pH 8.0. The clarified cell lysates were loaded onto the columns at 1 ml/min. Non-specifically bound material was washed off the column at 1 ml/min with 10 column volumes of buffer containing 8 M urea, 1 mM β-mercaptoethanol, 500 mM NaCl, 5 mM imidazole and 20 mM sodium phosphate, pH 8.0. The denatured recombinant proteins bound onto the column were refolded on-column at a flow rate of 0.5 ml/min by gradually removing the urea from the buffer over 15 column volumes. The refolded recombinant proteins bound to the column were then washed at 1 ml/min with 10 column volumes of

buffer containing 500 mM NaCl, 5 mM imidazole, 20 mM sodium phosphate, pH 8.0 and 1 mM β -mercaptoethanol. Refolded recombinant proteins were eluted off the column at 1 ml/min by increasing the concentration of imidazole from 5 mM to 500 mM in 20 column volumes. Elution fractions (1 ml) were collected and analysed by SDS-PAGE followed by Coomassie staining. Fractions containing SLPI-WT or its SLPI-A16G and SLPI-S15G-A16G variants were pooled and dialysed at 4°C with gentle agitation against 10 volumes of PBS pH 7.4 with two changes of dialysis buffer. The concentrations of the purified recombinant proteins were determined by BCA assay according to the manufacturer's instructions (Pierce BCA Assay, Fisher Scientific UK, Leicestershire).

Characterisation of purified SLPI-WT, SLPI-A16G and SLPI-S15G-A16G variants by SDS-PAGE

Purified dialysed SLPI-WT and its SLPI-A16G and SLPI-S15G-A16G variants were analysed by SDS-PAGE on 15% (w/v) polyacrylamide gels under reducing Laemmli conditions. Gels were stained with Coomassie Brilliant blue for total protein analysis. For Western blotting, gels were transferred onto nitrocellulose membrane and the membranes blocked with 3% (w/v) BSA in PBS containing 0.1% (v/v) Tween 20 for 1 h at room temperature. Probing was carried out with a biotinylated anti-SLPI antibody (1:500 dilution; R&D Systems, Abingdon, UK) followed by incubation with streptavidin conjugated horseradish peroxidase (HRP) (1:2,500 dilution; BioLegend, London UK) in 3% BSA (w/v) PBS containing 0.1% Tween 20 or with rabbit anti-His antibody (1:1,000 dilution; Insight Biotechnology Ltd., Wembley, UK) followed by incubation with HRP conjugated goat anti-rabbit antibody (1:10,000 dilution; Insight Biotechnology Ltd., Wembley, UK). Peroxidase activity was detected using a chemiluminescent substrate (GE Healthcare, Buckinghamshire, UK) and analysed using the Syngene G:Box and GeneSnap software (SynGene UK, Cambridge).

Neutrophil elastase inhibition assays

Neutrophil elastase (NE; 100 ng; Elastin Products Company, Missouri, USA) was incubated for 10 min at 37°C alone or with different amounts of recombinant SLPI-WT, SLPI-A16G or SLPI-S15G-A16G variant in 80 µl (final volume) of 0.5 M NaCl, 100 mM HEPES pH 7.5, 0.1% (v/v) Brij 97. After addition of 20 µl of N-(methoxysuccinyl)-Ala-Ala-Pro-Val-7-amino-4-methylcoumarin (AAPV-AMC; 100 µM final concentration; Enzo Life Sciences, Exeter, UK), NE activity was monitored by detecting the release of the fluorescent AMC cleavage product (excitation at 360 nm ; emission at 465 nm) for 12 min at 37°C using a 96-well microplate reader (Synergy HT using Gen5™ software, BioTek, Bedfordshire, UK). The change in relative fluorescence units over a 12 min period were calculated for all samples and the results expressed as percentage of non-inhibited NE.

EMSA

The binding of SLPI-WT and the SLPI-A16G and SLPI-S15G-A16G variants to an NF-κB consensus DNA binding site was assessed by Electrophoretic Mobility Shift Assay (EMSA) as described previously^{10,25}. Recombinant SLPI-WT and SLPI-A16G and SLPI-S15G-A16G variants (1 µg) were incubated with double-stranded biotinylated NF-κB consensus oligonucleotide 5'-AGTTGAGGGGACTTTCCCAGGC-3' (100 pmol; Life Technologies, Paisley, UK) and Poly(dI-dC).Poly(dI-dC) (2 µg; Sigma-Aldrich) for 30 min at room temperature in binding buffer containing 4% (v/v) glycerol, 0.1 mg/ml nuclease-free BSA, 1 mM EDTA, 5 mM DTT, 100 mM NaCl and 10 mM Tris-HCl, pH 7.5 (20 µl). After incubation, the mixtures were

electrophoresed on native 15% polyacrylamide gels. The gels were transferred onto 1 µm pore size nitrocellulose membranes in 1 x TBE for 30 min at 380 mA, 100 V and then cross-linked under UV light for 10 min. A Chemiluminescent Nucleic Acid Detection Kit (Pierce, Fisher Scientific UK, Leicestershire) was used to detect the SLPI:DNA complexes which were analysed using the Syngene G:Box and GeneSnap software.

LPS binding assay

Binding of recombinant SLPI-WT and SLPI-A16G and SLPI-S15G-A16G variants to *P.*

aeruginosa LPS was assessed by indirect ELISA as previously described with some minor modifications^{10,33}. Briefly, Greiner® high binding 96 well plates were coated with serial dilutions (in serum-free medium) of recombinant SLPI-WT, SLPI-A16G and SLPI-S15G-A16G variants. Blank wells were coated with diluent alone. The plate was incubated at 37°C for 2 h and then washed three times with PBS containing 0.05% (v/v) Tween 20. The plate was blocked for 1 h at room temperature with 200 µl of 1% (w/v) BSA PBS containing 0.05% (v/v) Tween 20 per well. Biotinylated *P. aeruginosa* LPS (100 ng) was then added to each well and the plate was incubated at 37°C for 3 h. The plate was washed three times before addition of 100 µl per well of streptavidin-conjugated HRP (1:2,500 dilution). After incubation at room temperature for 20 min, the plate was washed and ABTS single solution substrate (Life Technologies, Paisley, UK) was added. The absorbance at 405 nm of the wells was measured on a Biotek Synergy HT plate reader and data presented for each SLPI protein are corrected with the background absorbance readings obtained from wells coated with diluent only.

Cystic fibrosis sputum and study approval

Sputum from *Pseudomonas* infected CF patients was obtained anonymously from the adult CF Centre at Belfast City Hospital. Sputum samples were in excess to requirements for diagnostic purposes. Permission to use sputum samples, which would have been disposed of, for validation purposes was given by the Director of R&D, Belfast Health and Social Care Trust. Sputum was frozen immediately at – 80 °C and used the next day after thawing at room temperature.

Western blot analysis of recombinant SLPI incubated with CF sputum and NE

Each purified SLPI protein (100 ng) was incubated with 10 µl of pooled *Pseudomonas*-positive CF sputum or 2,500 ng of NE in Tris-buffered saline (total volume of 20 µl) for 8 h at 37°C as previously described¹⁰. Samples were separated by denaturing SDS-PAGE using 15% polyacrylamide gels and blotted onto nitrocellulose membrane. Membranes were probed using biotinylated anti-SLPI antibody as described above, visualised and individual SLPI bands were analysed by densitometry using the Syngene G:Box and GeneSnap software.

Effect of SLPI-WT and SLPI-A16G in an in vivo model of pulmonary infection

Housing and experimentation was carried out in accordance with the Animal (Scientific Procedures) Act 1986 and current guidelines approved by the Queen's University Ethical Review Committee. A log phase culture of clinical strain of *P. aeruginosa* (PA Q502) isolated from the lungs of a CF patient was washed and suspended in sterile endotoxin-free PBS (Sigma-Aldrich) at an OD (600 nm) of 0.5, equating to 3×10^8 CFU. C57BL/6 mice purchased from Charles Rivers Laboratories were anaesthetized and intranasally inoculated with 20 µl of PA Q502 or saline control. Concurrently, 100 µg of SLPI-WT and SLPI-A16G (or saline control) was administered intraperitoneally. Animals were sacrificed 24 h post infection. The

animals were exsanguinated by cardiac puncture; the blood was coagulated at room temperature then centrifuged at 13,000 x g for 10 min. Sera was stored at -20°C until required for analysis. KC was quantified by ELISA (R&D Systems, Abingdon, UK) following the manufacturer's instructions.

Perfused, minced lungs were incubated in Iscove's Modified Dulbecco's Medium (IMDM) containing 1 mg/ml of Collagenase D and 200 µg/ml of DNase for 1 h at 37°C, 200 x g. The tissue was then passed through a 70 µm cell strainer resulting in a single cell suspension. The cells were washed with sterile PBS and red blood cells were lysed with 3 ml of ACK lysis buffer (NH₄Cl 8.3g/L, KHCO₃ 1g/L, 3.72g/L EDTA in distilled water). The cells were counted and incubated with Fc block (1:1000 in PBS, eBioscience, Hatfield, UK) for 15 minutes. The cells were stained for flow cytometry with CD45 FITC (1:1000 in PBS, BioLegend), CD11b APC (1:1000 in PBS, eBioscience), Gr1 PE (1:1000 in PBS, eBioscience), F4/80 PeCy7 (1:500 in PBS, eBioscience) for 20 minutes in the dark at room temperature. Cells were then washed and acquired using a FACSCanto II (BD Biosciences, Oxford, UK). The data was analysed using FlowJo software (Tree Star).

Statistical analysis

All data were analysed using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA) and are reported as mean ± SEM or median (IQR) where appropriate. Results are representative of $n = 3$ unless otherwise indicated. Means were compared by unpaired t -test, Mann Whitney test, one-way analysis of variance (ANOVA) or Kruskal-Wallis test as appropriate. $P < 0.05$ was accepted to indicate statistical significance.

Supplementary Figures

Supplementary Figure 1: **Effect of SLPI-WT and SLPI-AG on lung cytokine and chemokines levels in a mouse model of *Pseudomonas aeruginosa* infection.** C57BL/6 mice received an intranasal administration of saline or *P. aeruginosa* Q502 (PA), in combination with intraperitoneal saline, SLPI-WT or SLPI-A16G ($n = 5$ /group). After 24 h, levels of MIP-2, IL-1 β , TNF α , KC and IL-6 were measured in lung homogenate by ELISA.

Supplementary Figure 2: **Effect of SLPI-WT and SLPI-AG on serum cytokine and chemokines levels in a mouse model of *Pseudomonas aeruginosa* infection.** C57BL/6 mice received an intranasal administration of saline or *P. aeruginosa* Q502 (PA), in combination with intraperitoneal saline, SLPI-WT or SLPI-A16G ($n = 5$ /group). After 24 h, levels of MIP-2, IL-1 β and IL-6 were measured in serum by ELISA.

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Figure Legends

Figure 1. Mutation of neutrophil elastase cleavage sites in recombinant SLPI. (A) The relative amino acid frequency at the P1 position of neutrophil elastase (NE) cleavage sites identified in human SLPI¹⁰ was determined using the MEROPS database²⁴. Gly was selected to replace the Ser¹⁵ and Ala¹⁶ residues at the P1 position in the cleavage sites of SLPI by NE as Gly is rarely found at the P1 position of NE substrates. **(B)** The positions of NE cleavage sites in SLPI are indicated by the arrows between Ser¹⁵-Ala¹⁶ and between Ala¹⁶-Gln¹⁷. The cleavage sites were mutated to Gly¹⁶-Gln¹⁷ for SLPI-A16G and Gly¹⁵-Gly¹⁶ for SLPI-S15G-A16G. The mutations are indicated in underlined bold type.

Figure 2. SDS-PAGE analysis of purified SLPI-WT, SLPI-A16G and SLPI-S15G-A16G. SLPI variants were expressed as N-terminal His₆-tagged recombinant proteins within the pQE30 expression vector in M15 [pREP4] *E. coli* and purified by immobilized metal ion affinity chromatography. Purified SLPI-WT, SLPI-A16G and SLPI-S15G-A16G variants were analysed by SDS-PAGE on 15% (w/v) SDS-PAGE gels under reducing Laemmli conditions. **(A)** Gels were stained with Coomassie Brilliant blue for total protein analysis. **(B)** Proteins were transferred onto nitrocellulose membrane and SLPI detected with a biotinylated anti-SLPI antibody. **(C)** Proteins were transferred onto nitrocellulose membrane and SLPI detected with a rabbit anti-His tag antibody.

Figure 3. Binding of SLPI and the SLPI variants to DNA. SLPI-WT, SLPI-A16G and SLPI-S15G-A16G (1 µg) were incubated with a biotinylated consensus NF-κB DNA oligonucleotide, electrophoresed on a 15% polyacrylamide gel and transferred onto nitrocellulose

membrane. SLPI:NF- κ B oligonucleotide complexes were detected by incubating the blot with streptavidin-HRP and visualised using a chemiluminescent substrate.

Figure 4. LPS binding properties of SLPI variants. Serial dilutions of SLPI-WT, SLPI-A16G and SLPI-S15G-A16G variants were analysed via ELISA to determine the relative LPS binding properties of the purified proteins. Biotinylated *Pseudomonas aeruginosa* LPS bound to SLPI was calculated as the increase in absorbance at 405 nm. Data presented for each SLPI protein are corrected with the background absorbance readings obtained from wells coated with diluent only.

Figure 5. Susceptibility of purified SLPI to cystic fibrosis sputum and neutrophil elastase proteolytic cleavage. Recombinant SLPI-WT, SLPI-A16G and SLPI-S15G-A16G were incubated with a **(A)** 10-fold molar excess of NE or **(B)** pooled CF sputum for 8 hours at 37°C. Samples were analysed by SDS-PAGE and Western blotting with an anti-His tag antibody. Each result for **(C)** NE and **(D)** CF sputum were analysed by densitometry.

Figure 6. SLPI-A16G attenuates pulmonary neutrophil infiltration in a mouse model of *Pseudomonas aeruginosa* infection. C57BL/6 mice received an intranasal administration of saline or *P. aeruginosa* Q502 (PA), in combination with intraperitoneal saline, SLPI-WT or SLPI-A16G ($n = 5$ /group). After 24 h, **(A)** pulmonary neutrophil counts were characterised by flow cytometry as CD11b⁺Gr1⁺ and **(B)** serum KC levels were quantified by ELISA. * $P < 0.05$; ** $P < 0.01$ versus PA.

Table 1. Primers used for the cloning of SLPI-WT into the pQE30 expression vector and for the preparation of SLPI-A16G and SLPI-S15G-A16G by site-directed mutagenesis.

Primer DNA sequence	Primer name
TTTTTTGGATCCGGAAAGTCCTTCAAAGCTGGAGTCTGTC	SLPI-WT BamHI forward
TTTTTTAAGCTTATGCTTTCACAGGGGAAACGCAGGATTTCC	SLPI-WT HindIII reverse
GGAGTCTGTCCTCCTAAGAAATCTGGTCAGTGCCTTAGATAC AAGAAACCTG	SLPI-A16G forward
CAGGTTTCTTGTATCTAAGGCACTGACCAGATTCTTAGGAG GACAGACTCC	SLPI-A16G reverse
GGAGTCTGTCCTCCTAAGAAAGGTGGCCAGTGCCTTAGATAC AAGAAACCTG	SLPI-S15G-A16G forward
CAGGTTTCTTGTATCTAAGGCACTGGCCACCTTCTTAGGAGG ACAGACTCC	SLPI-S15G-A16G reverse

Figure 1

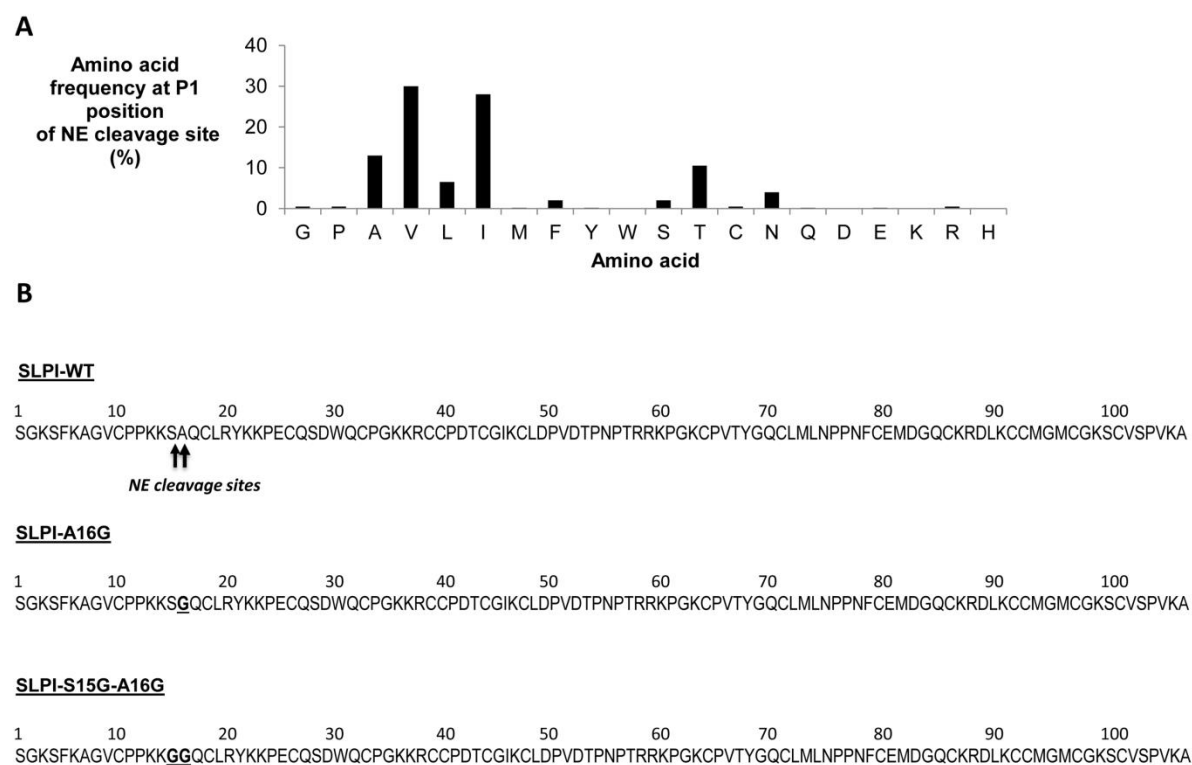


Figure 2

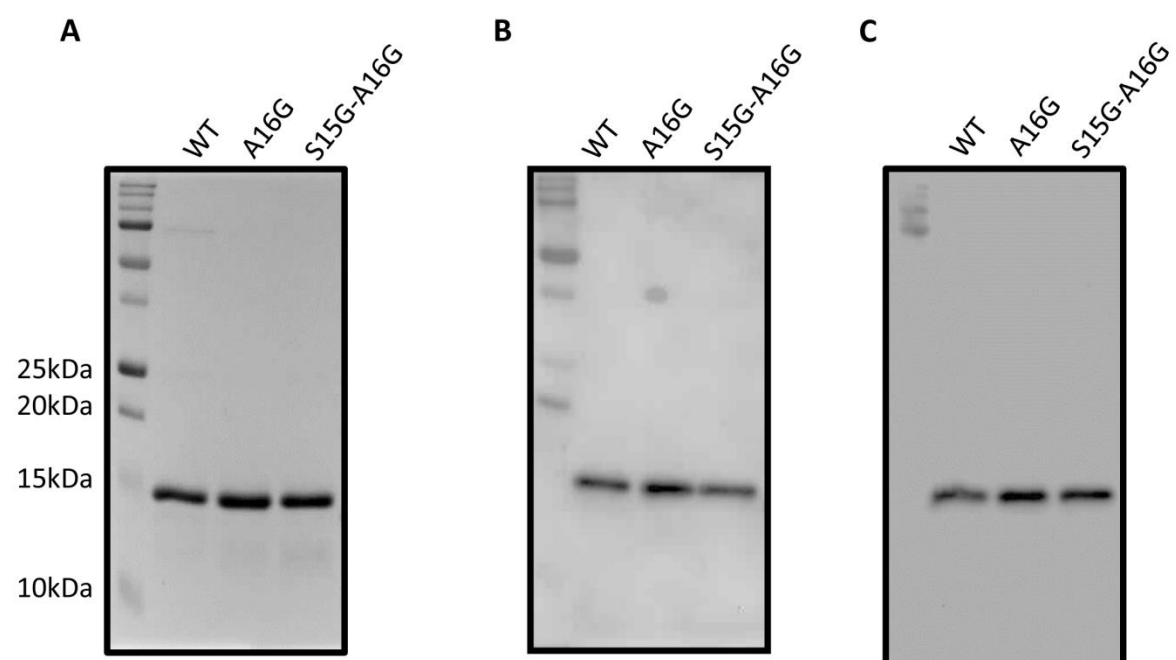


Figure 3

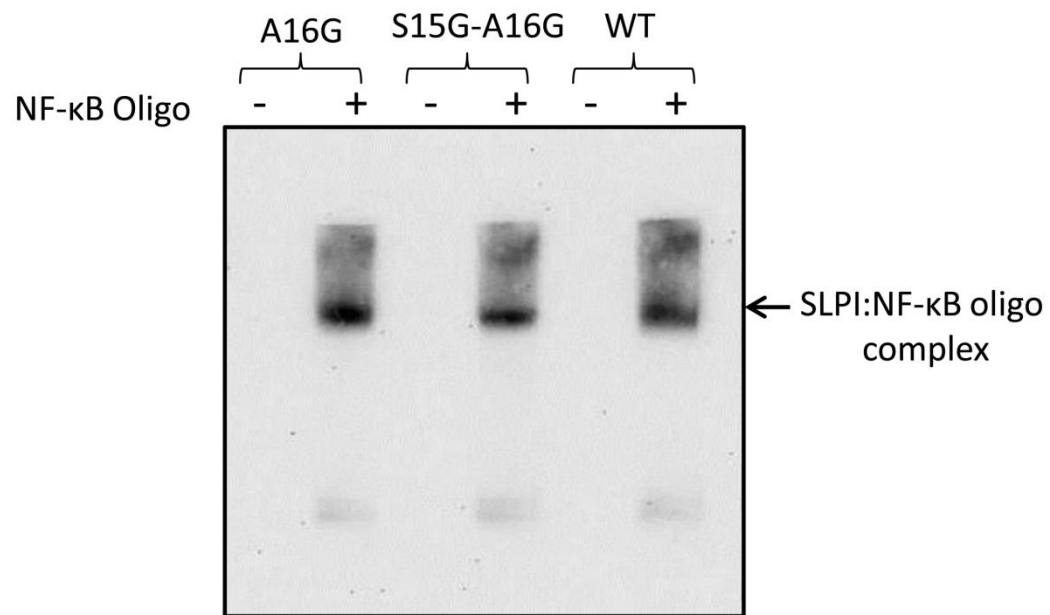


Figure 4

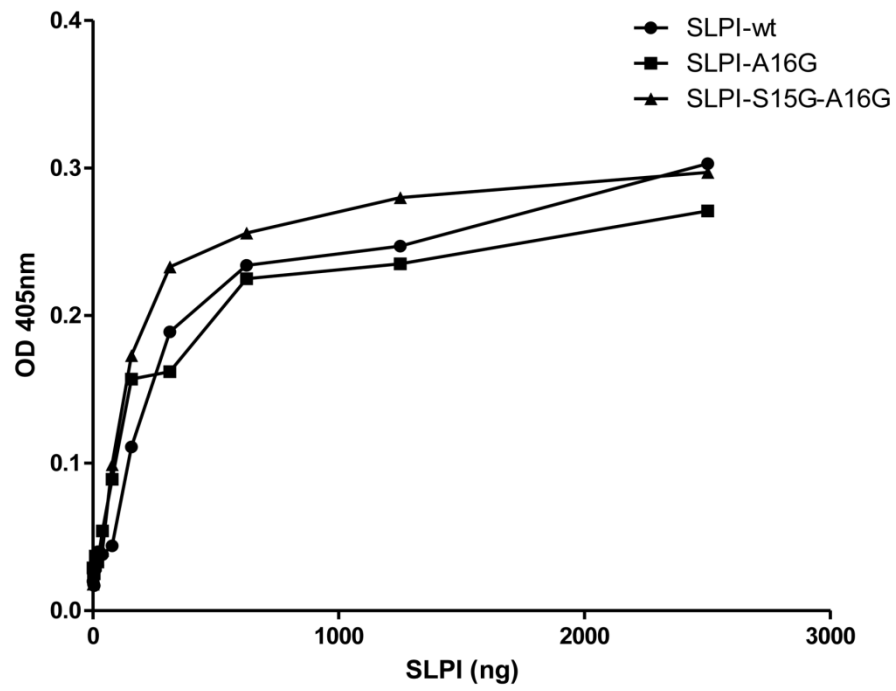


Figure 5

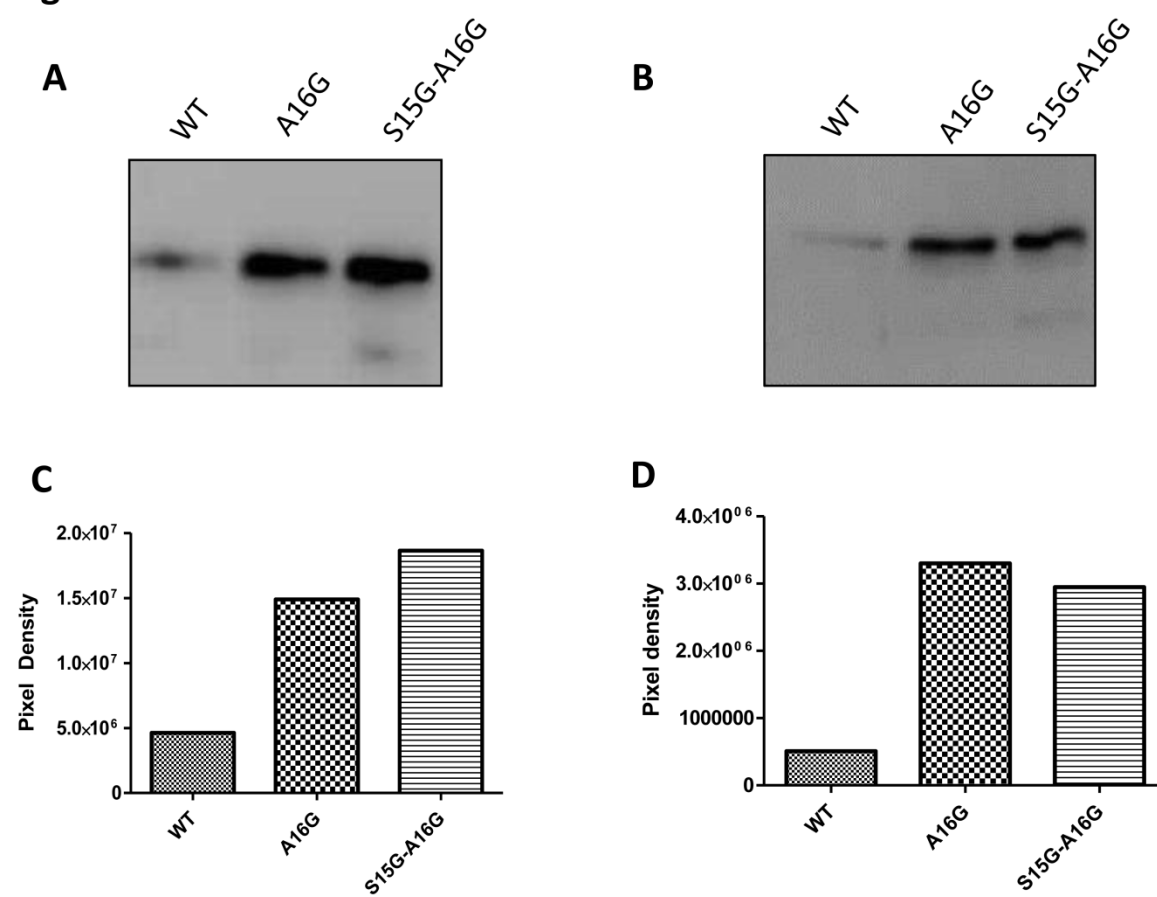
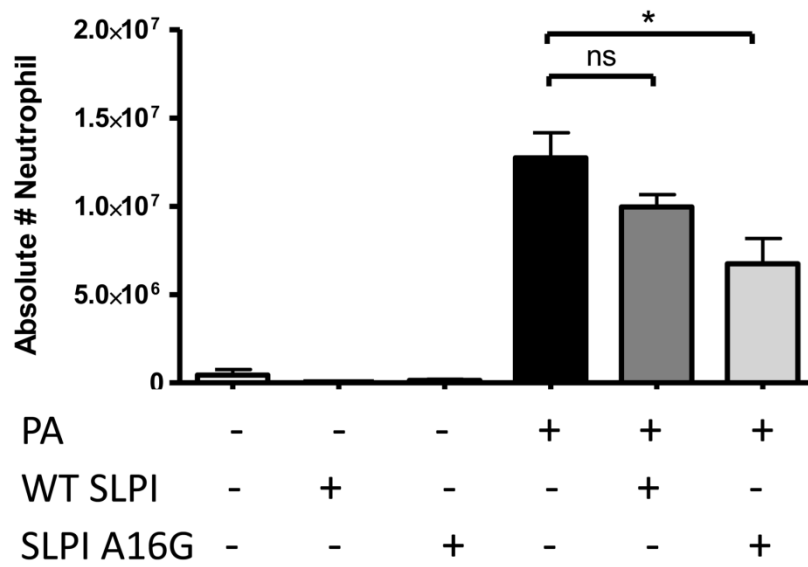
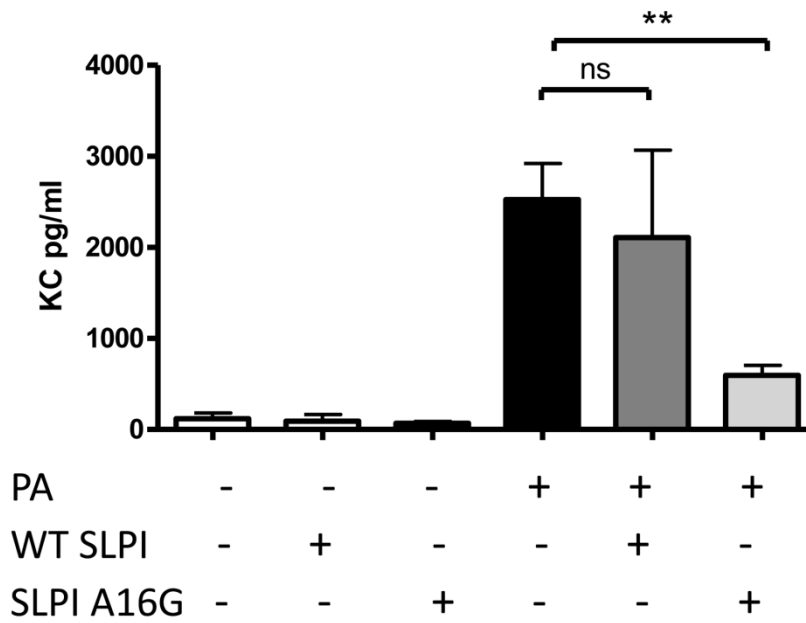


Figure 6

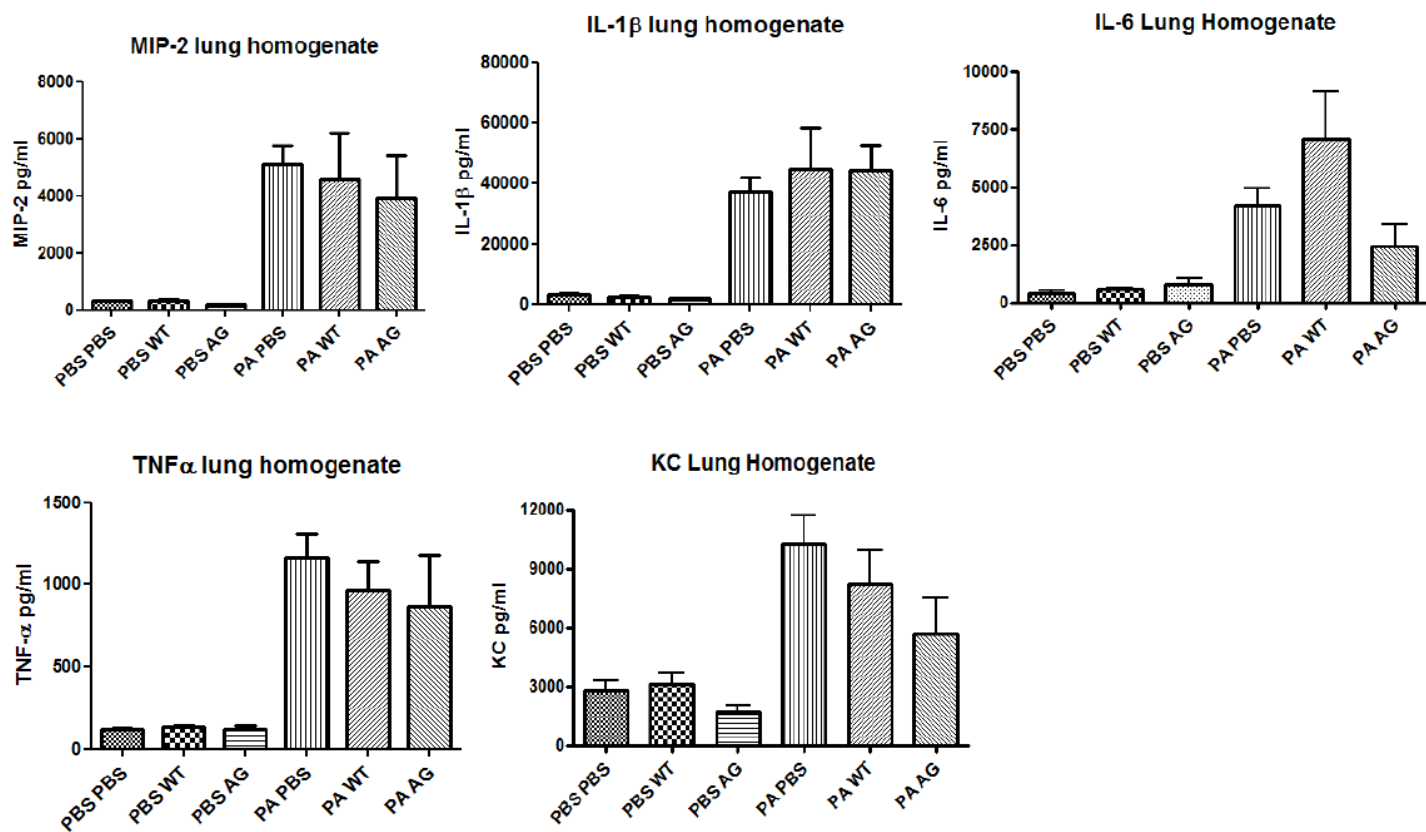
A



B



Supplement Figure 1



Supplement Figure 2

